Influence of phenolics on *in vitro* growth of *Frankia* strains

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The influence of some common plant phenolics was tested on six *Frankia* strains isolated from both *Alnus* and *Elaeagnus* host plants. The addition of 1 m*M* of different phenolics to QmodB broth significantly influenced the growth and (or) the morphological development of *Frankia*. Ferulic, *o*-coumaric, and *p*-coumaric acids were extremely effective in inhibiting the growth of *Frankia* colonies, increasing the ramification of hyphae, and decreasing the number and size of sporangia produced *in vitro*. However, benzoic and *p*-hydroxybenzoic acids did not influence the total growth of *Frankia* colonies but stimulated the *in vitro* production of spherical septate vesicles on the two strains of *Frankia* type N tested. The possibility that these active phenolics, which are known to be present in *Alnus* actinorhizae, might act as "chemical mediators" between the host cell and its endophytic *Frankia* is suggested.

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Les auteurs ont étudié l'influence de quelques substances phénoliques d'origine végétale sur six souches de *Frankia* isolées d'*Alnus* et d'*Elaeagnus*. L'addition au bouillon QmodB des diverses substances phénoliques à une concentration de 1 m*M* influence d'une manière significative la croissance et (ou) le développement morphologique de *Frankia*. Les acides férulique, *o*-coumarique et *p*-coumarique inhibent très efficacement la croissance des colonies de *Frankia*, ils augmentent la ramification des hyphes et diminuent le nombre et la dimension des sporanges produits *in vitro*. D'autre part, les acides benzoïque et *p*-hydroxybenzoïque n'influencent pas la croissance totale des colonies de *Frankia*, mais ils stimulent la production *in vitro* de vésicules sphériques cloisonnées chez les deux souches testées de *Frankia* de type N. Ces substances phénoliques actives, qui se rencontrent dans les actinorhizes d'*Alnus*, pourraient jouer le rôle de "médiateurs chimiques" entre la cellule de l'hôte et le *Frankia* endophyte.

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Introduction

Frankia is a slow-growing and pleomorphic actinomycete able to form nitrogen-fixing actinorhizae on a variety of host plants including *Alnus* sp., *Elaeagnus* sp., *Shepherdia* sp., *Casuarina* sp., and others (Torrey 1978).

The recent isolation and cultivation *in vitro* of numerous *Frankia* strains (Callaham *et al.* 1978; Quispel and Tak 1978; Baker, Kidd, and Torrey 1979; Baker, Torrey, and Kidd 1979; Berry and Torrey 1979; Lalonde and Calvert 1979; Lechevalier and Lechevalier 1979; Quispel 1979; Torrey *et al.* 1980; Lalonde *et al.* 1981; Diem *et al.* 1982; Normand and Lalonde 1982; Shipton and Burggraaf 1982) now make possible investigations on how an actinorhizal host plant and a *Frankia* endophyte interact by means of biochemical molecules.

During its probable life cycle as a free-living actinomycete in soil and (or) as an endophytic symbiont in the root tissues of an actinorhizal host, *Frankia* is probably exposed to some plant phenolics. Phenolics, which are frequently present in plant tissues (Harborne 1973), are often observed during the course of interaction between plants and microorganisms. The actinorhizal host plant *Alnus glutinosa* is known to contain large amounts of phenolics (Méndez *et al.* 1968). Li *et al.* (1969) demonstrated that phenolics contained in the roots of *Alnus rubra* inhibit the growth of the root parasite *Poria weirii*. By microscopic means, phenolics and (or) tannins were also frequently observed in the nodular tissues of various actinorhizal host plants after the infection of roots by a *Frankia* endophyte (Newcomb *et al.* 1978; Lalonde 1979). Blom (1981) tested tannin as a source of carbon for the *in vitro* growth of *Frankia* and found that it was not utilized. Dawson *et al.* (1981) demonstrated the potential inhibitory effect of juglone, a phenolic related compound, on *Frankia* sp. CpI1. Because nitrogen-fixing actinorhizal host plants, such as *Alnus* and *Elaeagnus*, will be interplanted with other species of trees, possible allelopathic interferences must be considered. Jobidon and Thibault (1981, 1982) studied the *Alnus–Frankia* symbiotic system in relation to *Populus*, a strong producer of phenolics. Using the strain *Frankia* sp. ACN1^{AG}, they suggested that *Frankia* can protect its *Alnus crispa* host plant against the inhibitory effects of the phenolics produced by *Populus balsamifera*.

To determine the effect of some common plant phenolics on *Frankia*, six different strains were exposed *in vitro* to solutions of some common plant phenolics.

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TABLE 1. Description of *Frankia* strains exposed to phenolics

	Strain acronym	Host plant	Origin	Reference
Type N[a]	$ACN1^{AG}$	*Alnus crispa*	Quebec	Lalonde and Calvert 1979
	$AGN1_g$	*Alnus glutinosa*	Holland	Lalonde *et al.* 1981
	$EAN1_{pec}$	*Elaeagnus angustifolia*	Ohio	Lalonde *et al.* 1981
	$EUN1_f$	*Elaeagnus umbellata*	Illinois	Lalonde *et al.* 1981
Type P[a]	$AR_gP5^{\underline{AG}}$	*Alnus rugosa*	Quebec	Normand and Lalonde 1982
	$AVP3_d$	*Alnus viridis*	France	P. Normand and M. Lalonde, unpublished data

[a]Type-P and type-N strains according to Normand and Lalonde (1982). Type P, spore positive; type N, spore negative.

The *Frankia* strains used were isolated from various *Alnus* and *Elaeagnus* species.

Materials and methods

The *Frankia* strains treated with phenolics are listed in Table 1. All strains were grown in glass tubes (20 × 150 mm) containing 17 mL of QmodB liquid medium supplemented with 5 mg/L of L-α-lecithin (Lalonde and Calvert 1979). During initial testing to find an appropriate solvent for the phenolics, ethanol and methanol were rejected because they significantly influenced sporogenesis of all six *Frankia* strains tested. However, dimethyl sulfoxide (DMSO) was found to have no apparent side effects on the growth of *Frankia* at concentrations less than 1% v/v. Consequently, phenolics were added at final concentrations of 0.1 m*M* and 1.0 m*M* of phenolics with a final DMSO concentration of not more than 0.42% v/v. All DMSO phenolic solutions were sterilized with a 0.2-μm Millex (Millipore, U.S.A.) filter. Five tubes per treatment were used. Each tube was inoculated with 0.5 mL of a *Frankia* suspension prepared from 20- to 30-day-old colonies grown in a single tube of the QmodB liquid medium. The *Frankia* suspension was prepared with a plastic syringe according to Lalonde and Calvert (1979). All tubes were incubated at 27°C without agitation.

The pH and osmotic concentration were measured (Osmette 2007, Precision Scientific) after the addition of the DMSO phenolic solutions, and then the tubes were inoculated with the diverse *Frankia* suspensions.

Frankia growth was measured using total protein content. The *Frankia* colonies of each tube were washed in phosphate-buffered saline solution (PBS, pH 7.2) (Lalonde and Calvert 1979), centrifuged, and resuspended in 800 μL of PBS. After 5 min of sonication (Fisher Sonic Dismembrator 300) in disposable plastic microtubes (Sigma, U.S.A.), the total protein content was evaluated on 100-μL aliquots, using the Bio-Rad protein assay (Bio-Rad, U.S.A.). Total protein content was determined at different times after inoculation. The growth values are expressed in micrograms per tube and represent the mean value of at least three replicates.

After different lengths of incubation, the *Frankia* colonies were sampled with a glass Pasteur pipette and observed without staining by interference contrast microscopy with an Ortholux II light microscope (Leitz-Canada) using 16, 40, and 100× objectives.

The absence of turbidity in the QmodB medium, the microscopic observation of all the cultures, and the subinoculation on six media (M standard method broth, BBL; malt agar, Difco; M-PH medium, BBL; standard method agar, BBL; potato dextrose agar, BBL; Czapek solution agar, Difco) were the criteria used for the testing of contamination.

After 30 days of incubation with phenolics, the *Frankia* colonies were used in an inoculation test of *Alnus glutinosa* seedlings grown in plastic growth pouches containing N-free Crone's nutrient solution according to Lalonde (1979).

Four weeks after inoculation with a *Frankia* suspension, the root systems of the *Alnus glutinosa* seedlings grown in plastic growth pouches were incubated without their shoot in 35 mL of air in glass vials containing 10% v/v of acetylene. After 30 min of incubation at room temperature, 0.2-mL samples were analysed in a gas chromatograph (Model 5710A, Hewlett Packard, U.S.A.) equipped with a flame ionization detector and a 2-mm × 1.8-m Porapak N, 80–100 mesh, filled column.

Results

Figure 1 illustrates the typical growth curve of *Frankia* sp. $ACN1^{AG}$ based on the total protein content of *Frankia* colonies. The mean protein content of *Frankia* growing in QmodB liquid medium is reproducible. Total protein content clearly indicated differences in the growth rates of the various *Frankia* strains tested (Table 2). The *Frankia* strains $EUN1_f$ and $EAN1_{pec}$, both isolated from *Elaeagnus* host plants, produced more total mean protein than the *Frankia* strains isolated from *Alnus* host plants.

Results of the microscopic observations of colonies of the *Frankia* sp. $ACN1^{AG}$ by interference contrast optics are summarized in Fig. 1 and illustrated in Fig. 2. The development of septate and slightly ramified hyphae occurs from the time of inoculation up to the end of incubation. A few spherical and septate vesicles can occur from the 2nd or 3rd day up to the 15th day of growth. For the same growth conditions and age of colonies, the abundance of vesicles produced *in vitro* differs substantially among *Frankia* strains. In this regard, *Frankia* sp. $EAN1_{pec}$ is one of the heaviest producers of vesicles in QmodB medium. The sporangia

TABLE 2. Mean protein contents (micrograms per tube) of six isolates of *Frankia* (host plant of origin, *Alnus* or *Elaeagnus*) grown on QmodB broth supplemented with phenolics at a final concentration of 0.1 m*M* and 1 m*M*

Compound	Concentration, m*M*	*Alnus* spp.					*Elaeagnus* spp.			
		Strain P[a]		Strain N[a]			Strain N[a]			
		AR_gP5^{AG}	$AVP3_d$	$ACN1^{AG}$	$AGN1_g$		$EAN1_{pec}$		$EUN1_f$	
		50[b]	50[b]	30[b]	37[b]	103[b]	35[b]	103[b]	35[b]	103[b]
Control[c]		19.6	40.6	80.0	61.8	471.7	195.0	478.3	519.0	1053.3
Benzoic acids										
Benzoic	1.0	17.5	8.4*	62.0	41.3	359.7*	260.5	373.3	122.0*	248.8*
	0.1	20.6	24.5	ND	77.8	462.3	255.5	463.0	520.0	1287.8
p-Hydroxybenzoic	1.0	21.4	6.0*	73.0	52.3	461.3	222.0	337.7	578.0	1226.1
	0.1	19.6	44.5	ND	59.8	465.7	182.0	485.3	634.0	1292.9
Cinnamic acids										
o-Coumaric	1.0	9.8*	7.0*	35.0*	11.3*	15.0*	15.0*	15.3*	80.5*	42.7*
	0.1	17.5*	19.5*	ND	48.0	251.0*	221.0	168.7*	260.0	207.7*
p-Coumaric	1.0	0.9*	0.2*	6.0*	5.5*	69.0*	0.5*	31.3*	10.0*	17.2*
	0.1	6.3*	33.7	ND	38.0	438.7	5.0*	48.5*	74.0*	11.9*
Ferulic	1.0	1.2*	23.0*	45.0*	26.5*	329.0*	0.5*	249.0*	70.0*	41.2*
	0.1	25.0	31.9	ND	48.0	479.0	242.0	553.7	512.0	555.7
trans-Cinnamic	1.0	18.7	7.3*	27.0*	35.0*	274.2*	294.0	251.3*	562.0	1004.2
	0.1	18.3	9.4*	ND	50.0	384.0	195.0	476.3	ND	954.2

NOTE, *, significantly lower than control for same column at the 95% confidence level; ND, not determined.
[a]According to Normand and Lalonde (1982).
[b]Incubated at 27°C for the specified number of days.
[c]In the control, equivalent amounts of 0.42% v/v DMSO added as for DMSO solutions of test compounds.

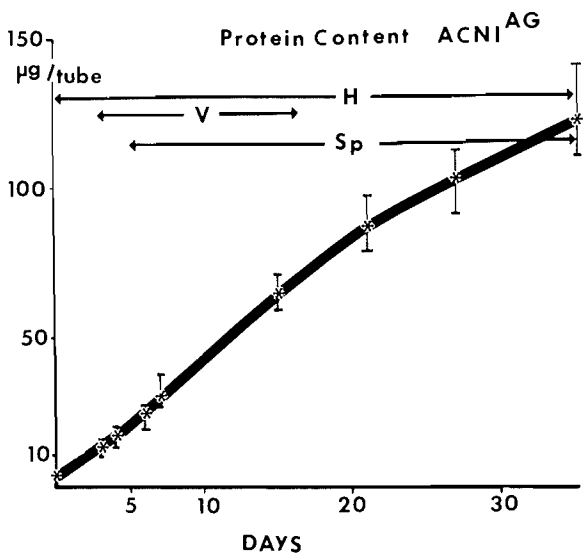

FIG. 1. Growth curve and morphogenesis of *Frankia* sp. $ACN1^{AG}$. Tubes of QmodB medium were inoculated at a density of 2.5 µg protein/tube from a 30-day-old culture of *Frankia* and then incubated at 27°C without agitation. The plotted points of total protein content represent means ± SD, $N = 4$. V, vesicles; H, hyphae; and Sp, sporangia.

appear from the 5th day up to the end of the incubation period with mature spores being liberated into the growth media more extensively after 15 days of incubation. Under the same conditions of growth and age of the colonies, the size of the mature sporangia is relatively constant within a particular *Frankia* strain but differs substantially between strains.

The growth of *Frankia* does not significantly influence the pH or the osmotic concentration of the QmodB liquid medium, nor does the addition of 1 m*M* concentration of any one of the six phenolic compounds tested (Table 3).

The four cinnamic acids evaluated were found to be more inhibitory to the growth of the *Frankia* strains, as determined by the total protein content, than the two benzoic acids (Table 2). This also shows that the four cinnamic acids were significantly inhibitory to the growth of *Frankia* when used at a concentration of 1 m*M*. Although showing a drastic difference in their growth rate, the different *Frankia* strains, originating from both *Alnus* and *Elaeagnus* and representing both type-N (spore-negative) and type-P (spore-positive) strains (Normand and Lalonde 1982), were all highly inhibited at a 1-m*M* concentration of three of the four cinnamic acids used, i.e., *o*-coumaric, *p*-coumaric, and

TABLE 3. Effect of 1-m*M* concentration of phenolic on the pH and osmotic concentration of the QmodB liquid medium inoculated with *Frankia* sp. ACN1^{AG} (sampled at 0 and 30 days)[a]

	pH		milliosmoles	
	0 days	30 days	0 days	30 days
Control[b]	6.38	6.60	158	169
Benzoic acids				
Benzoic	6.10	6.44	152	165
p-Hydroxybenzoic	6.04	6.40	158	174
Cinnamic acids				
o-Coumaric	6.20	6.32	161	177
p-Coumaric	6.02	6.18	156	180
Ferulic	6.04	6.32	158	175
trans-Cinnamic	6.10	6.37	183	197

[a]The tubes of QmodB medium were sampled at time 0, inoculated with a *Frankia* suspension, incubated at 27°C for 30 days, then sampled again for pH and osmotic concentration.
[b]In the control, equivalent amounts of 0.42% v/v DMSO added as for DMSO concentrations of test compounds.

TABLE 4. Relative effect of phenolic on the morphological development of *Frankia* strains ACN1^{AG} and AGN1$_g$[a]

Compound	Hyphae	Vesicles	Sporangia
Control[b]	Normal	Normal	Normal
Benzoic acids			
Benzoic	Normal	More numerous	Normal
p-Hydroxybenzoic	Normal	More numerous	Normal
Cinnamic acids			
o-Coumaric	More ramified	Normal	Smaller and less numerous
p-Coumaric	More ramified	Normal	Smaller and less numerous
Ferulic	More ramified	Normal	Smaller and less numerous
trans-Cinnamic	More ramified	Normal	Smaller and less numerous

[a]The two *Frankia* strains were grown for 30 days at 27°C on QmodB media supplemented with phenolic at a final concentration of 1 m*M*.
[b]In the control, equivalent amounts of 0.42% v/v DMSO added as for DMSO solutions of test compounds.

ferulic. The inhibitory effect of the fourth acid, i.e., *trans*-cinnamic, was more variable among the six *Frankia* strains tested.

In addition to their significant inhibition of growth of the six *Frankia* strains, the four cinnamic acids were found to alter drastically the morphology of the *Frankia* colonies (Table 4). The presence of any of the cinnamic acids in the QmodB broth was associated with a significant increase in the number of ramifications of the hyphae, in *Frankia* strains ACN1^{AG} and AGN1$_g$ (Figs.

FIG. 2. Interference contrast microscopy of *Frankia* sp. ACN1^{AG} grown at 27°C for 25 days in QmodB medium supplemented with 0.42% v/v of dimethyl sulfoxide. Hyphae (*h*) and large sporangia (Sp) can be seen. Bar is 10 μm. FIG. 3. Interference contrast microscopy of *Frankia* sp. ACN1^{AG} grown at 27°C for 25 days in QmodB medium supplemented with 1 m*M* of *p*-coumaric acid. Note the atypical hyphae (*h*) with numerous ramifications and the small sporangium (Sp). Bar is 10 μm. FIG. 4. Interference contrast microscopy of *Frankia* sp. ACN1^{AG} grown at 27°C for 25 days in QmodB medium supplemented with 1 m*M* of *trans*-cinnamic acid. Note the very small sporangia (Sp). Bar is 10 μm. FIG. 5. Interference contrast microscopy of *Frankia* ACN1^{AG} grown at 27°C for 25 days in QmodB medium supplemented with 1 m*M* of benzoic acid. Note the numerous vesicles (V) produced *in vitro*. Bar is 10 μm.

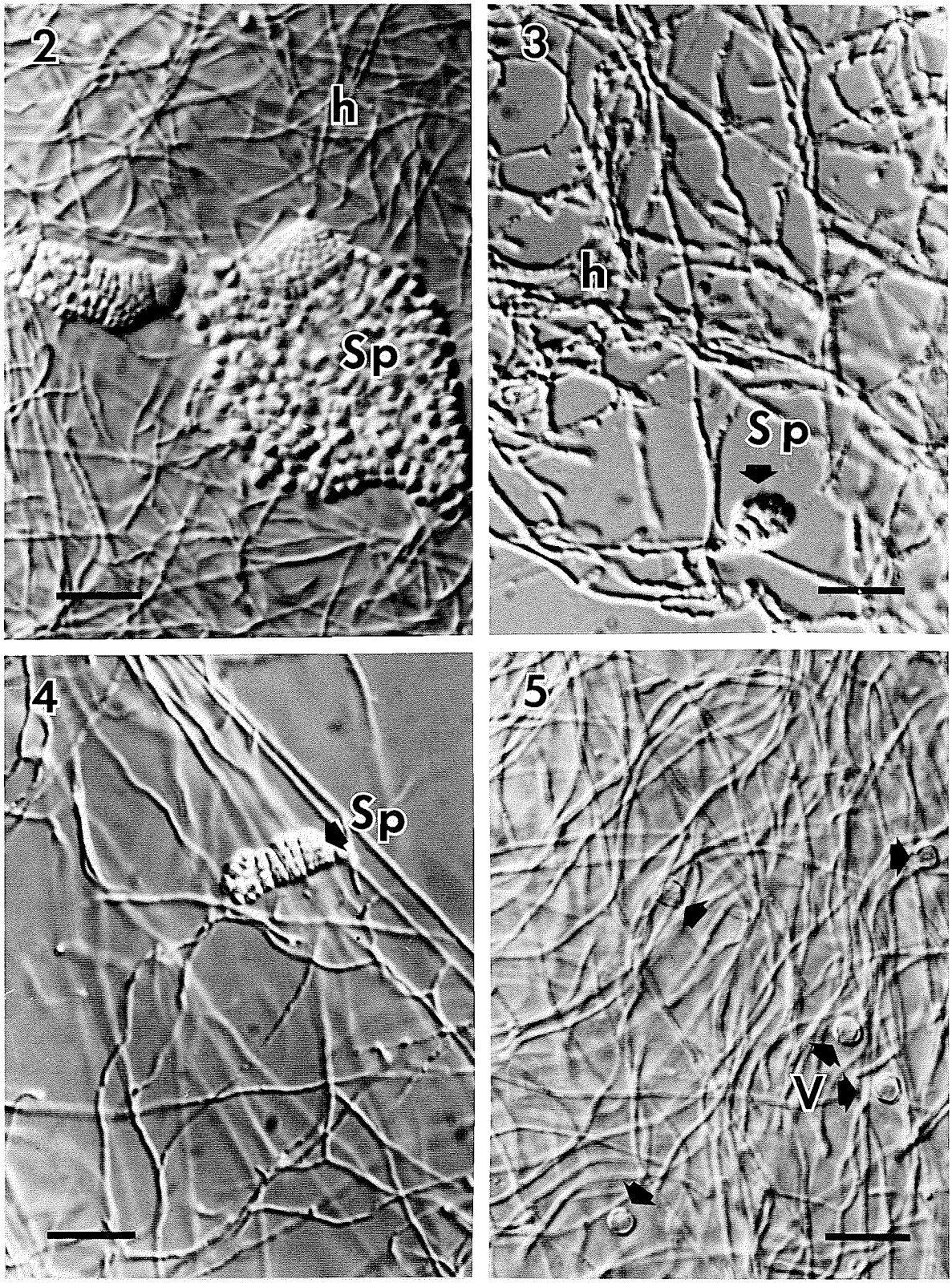
2
h
Sp
3
h
Sp
4
Sp
5
V

TABLE 5. Infectivity and effectivity of *Frankia* sp. ACN1^{AG} after 30 days of incubation with phenolic[a]

	No. seedlings nodulated / no. seedlings inoculated[b]	% nodulation	Effectivity[c]
Controls			
QmodB	34/34	100	+
QmodB + DMSO[d]	20/20	100	+
Benzoic acids			
Benzoic	18/18	100	+
p-Hydroxybenzoic	16/16	100	+
Cinnamic acids			
o-Coumaric	8/8	100	+
p-Coumaric	11/11	100	+
Ferulic	21/21	100	+
trans-Cinnamic	16/16	100	+

[a]The tubes of QmodB media were incubated at 27°C without agitation.
[b]The *Alnus glutinosa* seedlings were grown in plastic growth pouches supplemented with N-free Crone's nutrient solution according to Lalonde and Calvert (1979).
[c]Effectivity was measured as acetylene-reduction activity; + indicates presence of acetylene-reduction activity.
[d]An amount of 0.42% v/v DMSO was added as for DMSO solutions of test compounds used at a concentration of 1 m*M*.

3 and 4). A concentration of 1 m*M* of each of the cinnamic acids was also associated with significant and reproducible changes in sporulation, i.e., the sporangia being less numerous and smaller than normally formed when growing in the QmodB broth. However, the formation of spherical and septate vesicles remained normal for both *Frankia* strains in the presence of the four cinnamic acids tested at a concentration of 1 m*M* (Table 4).

Only three of the six *Frankia* strains evaluated were significantly inhibited by the highest concentration of both benzoic and *p*-hydroxybenzoic acids. These two benzoic acids were also noneffective in altering the normal growth of the *Frankia* hyphae as opposed to the cinnamic acids. Nevertheless, at a concentration of 1 m*M*, both benzoic acids were found to influence significantly the formation of spherical and septate vesicles in *Frankia* strains ACN1^{AG} and AGN1$_g$ (Table 4). Microscopic observations of the colonies exposed to the benzoic acids indicated a significant increase in the number of vesicles produced (Fig. 5). Furthermore, in the presence of benzoic acids, the increased formation of vesicles occurred 15–30 days after inoculation of the cultures, as opposed to the untreated controls where the fewer vesicles produced appeared 2–3 days after inoculation (Fig. 1).

Table 5 summarizes the tests of infectivity and effectivity of the *Frankia* strain ACN1^{AG} after its cultivation *in vitro* in the presence of the six different phenolic acids. After exposure of 30 days to either of the two benzoic acids or the four cinnamic acids, this *Frankia* strain isolated from *Alnus* was still as infective and effective as the control cultures not exposed to the phenolics and (or) to the DMSO solvent. The effectivity of the nodules, as determined by the acetylene-reduction assay, had a mean activity of 0.5–15 μmol ethylene $h^{-1} \cdot g$ ovendry weight of nodules^{-1}. Microscopic observations of the *Alnus* nodules induced by the phenolic-treated *Frankia* strain confirmed the presence of numerous typical spherical, septate vesicles inside the infected cortical cells of the host.

Discussion

The addition of 1 m*M* of different phenolics to the QmodB liquid medium significantly influenced the growth and (or) the morphological development of the six *Frankia* strains tested. The slight variations in pH and (or) osmotic concentration of the QmodB broth accompanying these additions cannot explain the observed effects.

The benzoic acids and cinnamic acids interfered quite differently on *Frankia*, although they differed only in type and position of radicals on a common benzene ring. The three cinnamic acids (*o*-coumaric, *p*-coumaric, and ferulic acids) were extremely effective in inhibiting the growth of *Frankia* colonies, increasing the ramification of hyphae, and decreasing the number and size of sporangia produced. The two benzoic acids (benzoic and *p*-hydroxybenzoic acid) did not significantly influence the total growth of *Frankia* colonies but were extremely effective in stimulating the production of spherical septate vesicles by the *Frankia* strains ACN1^{AG} and AGN1$_g$.

A similar increase in the number of vesicles produced *in vitro* was also achieved by Tjepkema *et al.* (1980) and Gauthier *et al.* (1981) on various *Frankia* strains, by the manipulation of the chemical components of the growth

media. Under the appropriate cultural conditions (e.g., P_{O_2}, carbon source, nitrogen content) induction of vesicles produced *in vitro* was associated with nitrogenase activity of the *Frankia* colonies. Nevertheless, our phenolic-induced vesicles were probably not active in fixing nitrogen owing to the high level of nitrogenous compounds in the QmodB medium.

The fact that all six *Frankia* strains tested reacted similarly in being inhibited in their growth in the presence of the *o*-coumaric, *p*-coumaric, and ferulic acids suggests some common physiological susceptibility between *Frankia* strains isolated from the two host specificity groups *Alnus* and *Elaeagnus*. Unfortunately, because the two *Frankia* strains $EAN1_{pec}$ and $EUN1_f$, both isolated from *Elaeagnus* spp., normally produce numerous vesicles when grown in the QmodB medium (Lalonde *et al.*1981), their susceptibility to the benzoic acids became impossible to evaluate.

Within the *Alnus* group of actinorhizal host plants, the type-N and type-P *Frankia* strains (= "spore-negative" and "spore-positive" strains), are known to be genetically different as expressed by differences in their physiological and morphological characteristics (Van Dijk and Merkus 1976; Quispel and Tak 1978; Burggraaf *et al.* 1981; Normand and Lalonde 1982). For example, the *in vitro* sporulation of a type-N *Frankia* strain is fully inhibited by the presence of fetal bovine serum (FBS, 65 mL/L) in the QmodB medium (Lalonde and Calvert 1979). But the extensive sporulation of a type-P *Frankia* strain is not fully inhibited in the presence of similar amounts of FBS. In the present report both types of *Frankia* strains reacted similarly to the presence of the cinnamic acids in the growth medium by decreasing significantly their total growth as evaluated by their protein content. However, the susceptibility of the type-P strains to the benzoic acids, so effective in increasing the number of vesicles produced by the type-N strains isolated from *Alnus*, became impossible to evaluate owing to the minimal amount of biomass present, the compactness of the colonies, and the early presence of large and numerous sporangia.

Generally speaking a type-N *Frankia* strain isolated from *Alnus* will produce few vesicles and many sporangia when grown *in vitro*. This is the opposite of the behavior of the same strain grown as an endophyte inside the root tissue of a susceptible *Alnus* host plant (Van Dijk and Merkus 1976). Our results indicate that following addition of the appropriate phenolic acids to their colonies growing *in vitro*, the *Frankia* strains $ACN1^{AG}$ and $AGN1_g$ can be induced to mimic an endophytic morphological status, i.e., formation of numerous spherical septate and very ramified hyphae with few sporangia. Méndez *et al.* (1968) reported the presence of numerous phenolics and more specially of ferulic, *p*-coumaric, *trans*-cinnamic, and *p*-hydroxybenzoic acids in root tissue of *Alnus glutinosa*. These are the same phenolics that were found to be active on *Frankia*. Furthermore, Li (1974) also reported these phenolics in the roots and root nodules of *Alnus rubra*. Although compartmentalized in vacuoles if present at the appropriate concentration in the root tissue of the host plant, phenolics might act as "chemical mediators" in the growth regulation of the pleomorphic *Frankia* endophyte. This influence of host phenolics on a *Frankia* endophyte might happen during the initial stage of penetration of a growing root hair, during the extensive development of hyphae and vesicles in the host cortical cells, and during the latter stage of sporulation in the older tissue at the base of the root nodule.

For a complete understanding of the *Frankia* symbiosis it seems imperative to qualify and quantify the various phenolics that are present in other groups of actinorhizal host plants and to determine their physiological participation in the host regulation of the growth and morphological development of a *Frankia* endophyte.

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